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Exploiting the Catalytic Power of Enzymes for Oxy- and Amino-functionalization Reactions

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"*What is life worth if not to be given?*"

Paul Claudel

The things brought to Mary

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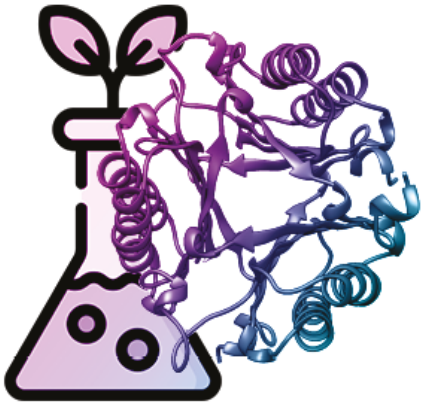
Aim and Outline



Aim and Outline of the Thesis

The worldwide environmental issues and global warming affecting our planet in the past decades urged the integration between chemistry and biology for constructing known and new molecules more efficiently and in a more environmentally friendly manner. The rising necessity of sustainable approaches in the chemical industry brought chemists and biochemists to get inspiration from Nature. Indeed, natural enzymes achieve excellent conversions and outstanding stereoselectivity in producing extremely diversified molecules. Using enzymes as "green" catalysts in chemical synthesis to build artificial biosynthetic processes has given rise to the scientific field nowadays known as biocatalysis. Single- and multi-step biocatalytic procedures, where one or more enzymes are involved, are applied under mild conditions, with high efficiency and selectivity, producing less waste. Thus, biocatalysis emerged as a valid alternative to traditional chemical processes in the past decades. In some instances, it has even become the method of choice, mainly to produce enantioenriched chiral compounds.[1,2] Nowadays, enzymes can be incorporated and utilized in chemical synthesis extensively because of the advances in DNA sequencing and synthesis technologies. The genomes of microorganisms and plants can be accessed and enable the mining of a vast number of proteins with different functions. Furthermore, techniques such as X-ray crystallography and cryo-electron microscopy allow scientists to unravel new enzyme structures and understand their catalytic mechanism by determining protein-ligand complexes. Informatic tools and artificial intelligence methods have also gained increased attention. For example, the AlphaFold 2 software enables the prediction of the tertiary structure of a specific amino acid sequence. This is highly beneficial when the respective crystal structure is not present yet or cannot be accessed.[3] Another helpful tool is the software RetroBiocat,[4] which simplifies and helps in the "retrobiosynthesis" of a complex molecule that is assembled backwards through its building blocks via enzymatic steps. Additionally, with the tremendous advances in laboratory evolution of enzymes to improve their capabilities, selectivity and robustness, and with all the recent advances in technologies to study the structure and mechanisms of enzymes, biocatalysis has great potential to become broadly and increasingly applicable in various industrial processes.

Oxygen and nitrogen incorporation within organic molecules by generating C-O and C-N bonds is extremely significant in chemical synthesis. The classical synthetic procedures for oxygenation and amination reactions often require high temperature and pressure, toxic solvents, and hazardous and cost-intensive compounds (e.g., transition metal complexes). For oxyfunctionalization reactions, a transition metal ion complex is normally required to activate molecular oxygen (O_2) and allow the transition from its ground state, where it exists as a triplet diradical ($^3O_2$) with a spin quantum number S=1, to its singlet spin state, where

its spin quantum number S=0. Therefore, dioxygen in its singlet state can react with organic molecules, which have also a spin quantum number S=0, producing oxygenated compounds. To allow the spin-forbidden reaction between dioxygen and organic molecules, the use of enzymes as sustainable alternative catalysts is of tremendous need.

Mono- and dioxygenases utilize O_2 as the oxygen source and often need an organic cofactor or a transition metal ion to transfer an electron to the dioxygen to generate the activated oxygen species able to react with common organic compounds. Heme-dependent mono- and dioxygenases, such as cytochrome P450 monooxygenases (CYPs), are among the most known and studied thus far. Other well-known cofactor-dependent monooxygenases are the Baeyer-Villiger and styrene monooxygenases, which utilize a flavin cofactor to activate molecular oxygen.[5] However, a significant number of oxygenating enzymes can allow the spin-forbidden reaction between dioxygen and organic molecules in a cofactor-independent manner.[6] The first cofactor-free monooxygenases discovered come from the biosynthetic pathway of polyketides and they present a ferrodoxin-like fold which is characterized by a β-α-β motif.[7,8] Among these enzymes, the most studied is ActVA-Orf6 from *Streptomyces coelicolor* and it is involved in the biosynthesis of a polyketide antibiotic named actinorhodin.[6] Another cofactor-less oxygenase, named 3,5-dihydroxyphenylacetyl-CoA 1,2-dioxygenase (DpgC), belongs to the crotonase superfamily and it is involved in the biosynthesis of the antibiotic vancomycin. The enzyme is characterized by a trimeric quaternary structure which helps the enzyme in stabilizing an enolate anion intermediate derived from acyl-CoA.[9,10] Other cofactor-independent oxygenases are known as 2,4-dioxygenases, belonging to the α,β-hydrolase-fold superfamily. These enzymes catalyze the degradation of the quinolone signaling molecules in bacteria. One of the best known in this class is named 1-H-3-hydroxy-4-oxoquinaldine-2,4-dioxygenase (Hod) and it is involved in the quorum sensing system of the pathogen *Pseudomonas aeruginosa*. The presence of a catalytic triad composed by a serine, a histidine and an aspartic acid allows a non-nucleophilic activation mechanism of molecular oxygen and its subsequent reaction with the organic substrate.[11,12] The catalytic mechanism of these peculiar class of enzymes is still under exploration. Since no cofactors nor metal ions are involved, the authors refer to their mechanism as "substrate assisted", meaning that the substrate plays the role of the cofactor in activating O_2. Therefore, the substrate needs to be able to transfer electrons easily and where a radical intermediate is involved, to stabilize the radical itself.[13] Although different hypotheses were proposed on the mechanism of cofactor-independent oxygenases, there are still several questions to be answered. Another unusual cofactor-less monooxygenase was discovered by Poelarends and coworkers in 2015 within the tautomerase superfamily, named RhCC. This unusual enzyme can catalyze the oxydative cleavage of 4-hydroxyphenylenolpyruvate (4-HPP).[14]

Organic peroxides, like hydrogen peroxide, are also convenient oxygen sources. Particularly, H_2O_2 is rather easy to handle and miscible with water. Furthermore, a direct electron transfer to the oxygen atom is not necessary since the oxygen atoms in the hydrogen peroxide are already reduced and the so-called *Oxygen Dilemma* can be overcome.[15] Enzymes able to bind organic peroxides for use in oxygenation reactions are known as peroxyzymes. For instance, CYPs can also utilize H_2O_2 especially for performing hydroxylations of inert C-H bonds, particularly important in late stage functionalization of complex natural products or pharmaceuticals.[16,17] Another interesting class of peroxyzymes are the copper-dependent lytic polysaccharides monooxygenases (LPMOs), able to convert recalcitrant polysaccharides, like chitin or cellulose, via hydroxylation of the glycosidic bonds. The LPMOs present an active site pocket containing a copper metal ion coordinated with three histidine residues.[18,19] Other important cofactor-dependent peroxygenases are the unspecific peroxygenases (UPOs) and the chloroperoxidases characterized by a heme thiolate cofactor. UPOs are particularly interesting because of their broad substrate scope, extracellular stability and high peroxygenase activities.[20] All the above-mentioned biocatalysts present an organic cofactor and/or a transition metal ion in the active site. Nonetheless, few examples of cofactor-independent peroxyzymes are known thus far, such as metal-free chloroperoxidases.[21] The 4-oxalocrotonate tautomerase (4-OT) was recently found able to accept different organic peroxides and catalyze the asymmetric epoxidation of cinnamaldehyde and cinnamaldehyde derivatives without the help of any organic cofactor nor metal ion.[22] In the past years, peroxygenases were largely studied and improved via protein engineering with different methods, due to their tremendous potential as promising biocatalysts for large scale synthesis.[23] The symmetry relationship within homohexameric 4-OT, with any point mutation reflected in all six subunits, makes its genetic optimization rather restricted. Recently, a tandem-fused version of 4-OT was developed, in which the C-terminus of one monomer and the N-terminus of the second monomer are connected via a short (five amino acids) flexible linker.[24] The tandem-fused 4-OT has a reduced symmetry compared to "unfused" wild-type 4-OT, expanding its genetic optimization potential by enabling independent sequence diversification of neighboring subunits, consequently enlarging the sequence space that can be sampled by directed evolution. The fused 4-OT was evolved to enhance its peroxygenation activity, enabling the efficient synthesis of α,β-epoxy-aldehydes in gram-scale with high enantiopurity.[25]

The enzymatic synthesis of linear and cyclic amines via, for example, transaminases, C-N lyases, or imine reductases is well-known and usually used as an environmentally benign substitute to standard chemocatalytic methodologies. Transaminases catalyze the reaction between a carbonyl substrate and an amine donor, while imine reductases commonly catalyze the asymmetric reduction of imines. Another interesting enzyme is the reductive aminase from *Aspergillus*

orizae, firstly discovered as an imine reductase homolog, that is able to catalyze the reductive amination between a carbonyl group (either an aldehyde or a ketone) and an amine.[26] Most recently other imine reductases were also found able to catalyze reductive amination reactions.[27] Another interesting group of enzymes are formed by the C-N lyases, which can be divided in three main subgroups: ammonia-lyases, amidine/amide-lyases and amine-lyases depending on the nature of their products. Among the subgroup of amine-lyases, ethylenediamine-*N*,*N*-disuccinic acid (EDDS) lyase (EDDS-lyase) is an attractive biocatalyst especially for the synthesis of L-aspartic acid derivatives. EDDS-lyase naturally catalyzes the degradation of (*S*,*S*)-EDDS via two sequential deamination steps, forming ethylenediamine and two equivalents of fumarate. However, it was demonstrated that it can also catalyze the reverse reaction, that is the formation of EDDS, becoming an appealing C-N bond-forming enzyme.[28] EDDS-lyase shares structural characteristics with aspartate/fumarate superfamily members and has a very broad nucleophile scope, which allows its application for the asymmetric synthesis of a wide variety of non-natural amino acids.[29–31]

To build molecular complexity out of simple building blocks, enzymatic artificial pathways can be constructed. Combining multiple biocatalysts in one pot presents several advantages such as reducing the number of reaction and downstream processing steps, giving less waste production and minimizing process time and costs. Water can be used as the sole reaction medium without involving the use of hazardous solvents. Moreover, the outstanding chemo- and stereoselectivity of the enzymes involved enable the production of chiral compounds with high enantiopurity. The incorporation of tailored enzymes gives a boost to produce relevant molecules via an efficient cascade route, as we underline in **chapter 1** and **chapter 2** of this thesis.

Overall, the research presented in this thesis aimed at studying and applying different C-O and C-N bond-forming enzymes, specifically cofactor-independent monooxygenases and peroxyzymes, as well as EDDS-lyase. Their potential as promising "green" catalysts is presented, either in single-step or multi-step cascade synthesis, to produce different groups of chiral organic molecules, like epoxides, aryl glycerols, and chiral non-natural amino acids as precursors to artificial sweeteners.

In **chapter 1** we give an outline of recently developed enzymatic cascades, where wild-type or engineered oxygenating and aminating enzymes are applied to build multi-step synthesis routes to achieve various molecular scaffolds. We stress the use of various peroxygenases for epoxidation and hydroxylation reactions and C-N bond-forming enzymes, such as C-N lyases, for the synthesis of non-natural amino acids. Numerous procedures with purified proteins and whole cell catalysts are described showing a wide variety of biocatalytic cascades and their potential for large-scale applications. In addition, the bottlenecks are underlined, and some new technologies, potentially able to overcome these issues, are presented.

Chapter 2 describes a two- and three-step enzymatic cascade for synthesizing enantiopure epoxides and aryl glycerol derivatives, with water as the only medium starting from renewable aldehydes. The system described combines three cofactor-less enzymes: an engineered aldolase, evolved from the promiscuous enzyme 4-oxalocrotonate tautomerase (4-OT), a peroxyzyme, evolved from a tandem-fused version of 4-OT and highly specific for hydrogen peroxide, and an epoxide hydrolase purposely selected based on literature data. Combining the evolved aldolase and the peroxyzyme gives access to enantioenriched α,β-epoxy-aldehydes as valid intermediates to further produce aromatic vicinal triols with high conversion, moderate yield and remarkable enantioselectivity, by coupling an epoxide hydrolase in the final step.

In **chapter 3** activity assays and structural studies of newly discovered putative cofactor-free 4-hydroxyphenylenolpyruvate (4-HPP) monooxygenases are reported. Although this research gave some interesting insights into the properties of this peculiar class of monooxygenases, there are still essential issues to be solved and their catalytic mechanism still needs to be fully elucidated. Therefore, their applicability as biocatalysts is still limited and needs further study.

In **chapter 4** we demonstrate the catalytic power of an engineered double mutant of EDDS-lyase, showing >1000-fold improvement in activity compared to wild-type EDDS-lyase for the biocatalytic enantioselective synthesis of the neotame (an artificial sweetener) precursor *N*-(3,3-dimethylbutyl)-L-aspartic acid. The elucidation of the EDDS-lyase mutant crystal structure in complex with two fumarate molecules gave access to docking studies explaining the role of the two mutations in binding the desired product in a favored manner.

Ultimately, **chapter 5** provides an overview of the whole thesis and proposes some future perspectives for studying and applying these fascinating biocatalysts for C-O and C-N bond formation.

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